New Metabolic Products of *Aspergillus flavus*. Part II.[1] Asperflavin, Anhydroasperflavin, and 5,7-Dihydroxy-4-methylphthalide

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Asperflavin, a yellow pigment from the culture filtrate of an entomogenous strain of *Aspergillus flavus*, is shown to be (+)-3,4-dihydro-3,6,9-trihydroxy-8-methoxy-3-methylanthracen-1(2*H*)-one (I; R = H). New minor metabolic products isolated were anhydroasperflavin (1,6-dihydroxy-8-methoxy-3-methyl-9-anthrone) (III; $R^1 = R^3 = H$, $R^2 = Me$) and a lactone, shown to be 5,7-dihydroxy-4-methylphthalide (VIII; $R^1 = R^2 = H$). The yellow spore-heads contained a mixture of the known fungal pigments aspergin (XII; R = n-C_5H_{11}·CH:CH·) and flavoglaucin (XII; R = n-C_7H_{15}).

THE isolation of a number of dihydroisocoumarins, the asperentins, by extraction with chloroform of the culture filtrate of an entomogenous strain of *Aspergillus flavus*, grown on synthetic media, has been described.[1] The crude extract also contained an unstable citrine phenolic pigment, $C_{16}H_{16}O_5$, to which the trivial name asperflavin is assigned. In the chromatographic separation of the asperentins, asperflavin was eluted with 5'-hydroxyasperentin; it was separated from the latter by fractional crystallisation and was obtained in a yield of 2 mg l^{-1}.

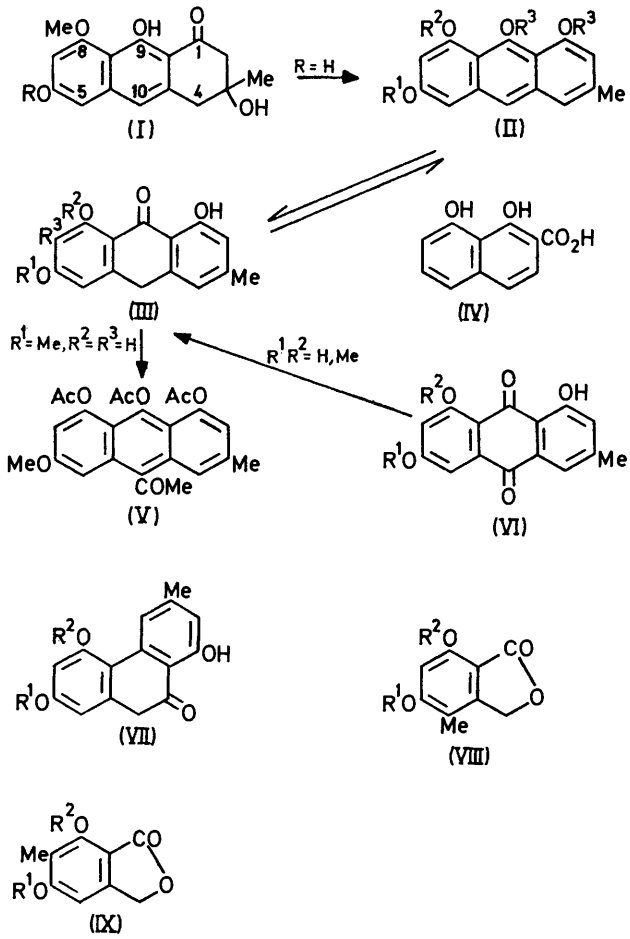
More polar substances with specific u.v. absorption remained in the culture filtrate after the initial extraction with chloroform; they were removed completely by further extraction with ethyl acetate. Chromatographic separation of this extract yielded small amounts (0·3 mg l^{-1}) of two compounds, an unstable, dimorphic, anhydroasperflavin, $C_{16}H_{14}O_4$, and a phenolic lactone, $C_9H_8O_4$.

These three new fungal metabolic products are now shown to be (+)-3,4-dihydro-3,6,9-trihydroxy-8-methoxy-3-methylanthracen-1(2*H*)-one (I; R = H), 1,6-dihydroxy-8-methoxy-3-methyl-9-anthrone (III; $R^1 = R^3 = H$, $R^2 = Me$), and 5,7-dihydroxy-4-methylphthalide (VIII; $R^1 = R^2 = H$), respectively. They were isolated from a fermentation designed to give maximum production of the asperentins and the yields quoted, particularly for asperflavin, are not necessarily the best attainable.

Asperflavin was optically active, $[\alpha]_D$ +4°. It contained a methoxy-group (τ 6·1) and a carbonyl group, which was chelated ($\nu_{max.}$ 1632 cm^{-1}), since asperflavin

[1] Part I, J. F. Grove, preceding paper.

gave an intense green colour with iron(III) chloride. The i.r. spectrum showed absorption bands at 3490 and 3350 cm^{-1}, attributed to two hydroxy-groups; neither



of these was the phenolic hydroxy-group involved in chelation since the monomethyl ether (I; R = Me) ($\nu_{max.}$ 3440 and 1635 cm^{-1}), obtained with methyl iodide in acetone in the presence of potassium carbonate, gave the same intense green colour with iron(III) chloride. It follows that asperflavin must contain three hydroxy-groups, two of which (one phenolic, involved in chelation, and one probably a tertiary alcohol) are not methylated under these conditions. All five oxygen atoms in asperflavin are thus accounted for.

[2] F. A. Hochstein, C. R. Stephens, L. H. Conover, P. P. Regna, R. Pasternack, P. N. Gordon, E. J. Pilgrim, K. J. Brunings, and R. B. Woodward, *J. Amer. Chem. Soc.*, 1953, **75**, 5455.

The u.v. spectrum of asperflavin (see Figure 1) was consistent with the presence of a 1,8-dihydroxynaphthalene chromophore conjugated with a C=O group,[2] which must then be in a third carbocyclic ring. The n.m.r. spectrum (see Table 1) showed the presence of a tertiary methyl group (τ 8·6), possibly contained in the partial

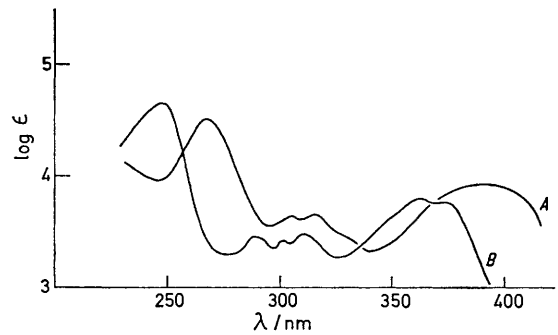


FIGURE 1 U.v. absorption curves for asperflavin *A* and 1,8-dihydroxynaphthoic acid *B*

structure $R^1R^2C(OH)Me$; three aromatic protons (τ 3·2, 3·4, and 3·5), two of which appeared to be *meta* coupled (*J* 2 Hz); and two-proton singlets (τ 6·9 and 7·2) in the region associated with benzylic protons. Pyrolysis of asperflavin, or mild acid-catalysed dehydration, gave anhydroasperflavin (III; $R^1 = R^3 = H$, $R^2 = Me$). This transformation was accompanied by the loss of both two-proton singlets from the n.m.r. spectrum of asperflavin, by a downfield shift of the tertiary methyl group to τ 7·7, by an increase in the number of aromatic ring protons, and by a marked hypsochromic shift in the u.v. absorption (see Figure 2).

Although this spectroscopic evidence was not inconsistent with the formation of a new aromatic ring, anhydroasperflavin nevertheless still contained a carbonyl group ($\nu_{max.}$ 1640 cm^{-1}) and gave a green iron(III) reaction similar to that of asperflavin. These facts can be accounted for by the formation of an anthrone (III) or phenanthrone (VII; R^1, R^2 = H or Me) system from the 9-anthrol (II) or 9-phenanthrol, initially produced by dehydration of asperflavin. The similarity of the u.v. absorption spectrum of anhydroasperflavin (see Figure 2) to that of the isomeric ' physcion anthranol B ',[3] shown on spectroscopic evidence (see Table 1) to exist as 1,8-dihydroxy-6-methoxy-3-methyl-9-anthrone (III; R^1 = Me, $R^2 = R^3 = H$) in both chloroform and methanol, suggested a linear annulation. This was confirmed when

TABLE 1

Chemical shifts (τ values) for protons in asperflavin and related compounds

Compound	Solvent	10-H	5,7-H	OMe	2,4-H_2	3-Me	OH
(I; R = H)	$(CD_3)_2CO$	3·22	3·38, 3·50	6·10	6,94, 7·18	8·62	−5·0, 3·72
	CD_3OD	3·25	3·50, 3·57	6·08	7·00, 7·22	8·64	3·82
(I; R = Me)	$CDCl_3$	3·0	3,3, 3·4	6·0	6·9, 7·0	8·6	−5·0
(III; $R^1 = R^2 = H$, R^3 = Me)	CD_3OD		3·54, 3·54	6·10	3,26, 3·26	7·70	
(III; R^1 = Me, $R^2 = R^3 = H$)	$CDCl_3$	5·75	3·60, 3·60	6·15	3·35, 3·35	7·65	

[3] J. N. Ashley, H. Raistrick, and T. Richards, *Biochem. J.*, 1939, **33**, 1291.

reduction of questin [4] (1,6-dihydroxy-8-methoxy-3-methylanthraquinone) (VI; $R^1 = H$, $R^2 = Me$) with zinc and acetic acid gave the 9-anthrone (III; $R^1 = R^3 = H$, $R^2 = Me$) identical with anhydroasperflavin.

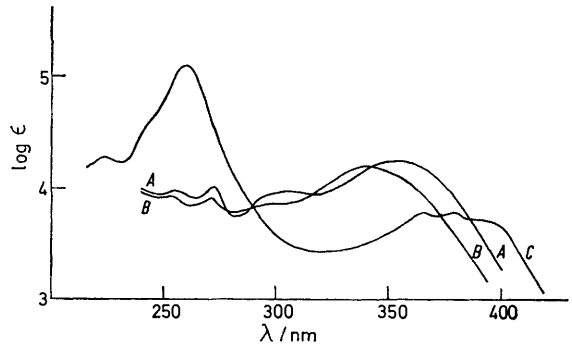
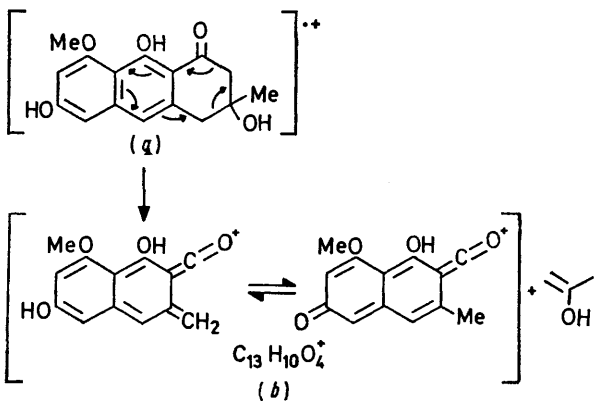


FIGURE 2 U.v. absorption curves for the anthrones (III; $R^1 = Me$, $R^2 = R^3 = H$) *A* and (III; $R^1 = R^3 = H$, $R^2 = Me$) *B*, and for the acetate (II; $R^1 = R^3 = Ac$, $R^2 = Me$) *C*

It follows that asperflavin has structure (I; R = H), a conclusion supported by the mass spectrum which contained an abundant resonance-stabilised rearrangement ion (*b*) at *m/e* 230 ($M^+ - C_3H_6O$). In the methyl

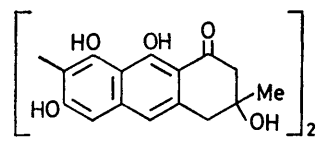


ether (I; R = Me), the corresponding ion (at *m/e* 244) was less stable and underwent further fragmentation with sequential loss of 15 and 28 mass units.

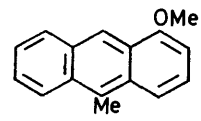
Because of their inherent instability, hydroxy-9-anthrones have been recorded only infrequently among fungal metabolites; phenol coupling reactions lead to more stable dimers. Known examples, all related to emodin (VI; $R^1 = R^2 = H$), are confined to the genus *Aspergillus* and include, in addition to anhydroasperflavin, the isomer (III; $R^1 = Me$, $R^2 = R^3 = H$) from numerous *Aspergillus* sp. of the *A. glaucus* series [3] and the chloro-derivative (III; $R^1 = R^2 = H$, $R^3 = Cl$) from *A. fumigatus*.[5] Flavomannin (X), a yellow pigment from the mycelium of *Penicillium wortmanii*,[6] is the dimer of an emodin anthrone hydrated in ring c. Asperflavin is the first monomeric compound of this type to be isolated.

Acetylation of anhydroasperflavin with acetic anhydride in pyridine at room temperature gave a yellow triacetate (II; $R^1 = R^3 = Ac$, $R^2 = Me$), derived from the tautomeric 9-anthrol: the u.v. absorption (Figure 2) was almost identical with that for the isomer (II; $R^1 = Me$, $R^2 = R^3 = Ac$) and similar to that reported [7] for 1-methoxy-10-methylanthracene (XI) [$\lambda_{max.}$ 239, 256, 356, 364, 375, and 396 nm (log ε 4·6, 5·02, 3·80, 3·75, 3·94, and 3·84). Acetylation of asperflavin under these conditions gave a complex mixture of products from which the monoacetate (I; R = Ac) and the triacetate (II; $R^1 = R^3 = Ac$, $R^2 = Me$) were separated by chromatography. Consistent with the greater stability of the anthrone tautomer in 1,8-dihydroxy-9-anthrones, *O*-acetylation of the anthrone (III; $R^1 = Me$, $R^2 = R^3 = H$) by this method was accompanied by *C*-acetylation giving the 10-acetyl derivative (V). The triacetate (II; $R^1 = Me$, $R^2 = R^3 = Ac$) was obtained with boiling acetic anhydride.[8]

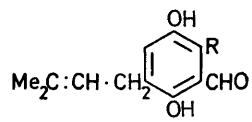
Thin layers of silica impregnated with asperflavin changed colour from citrine to green when exposed to light and air. As the methyl ether (I; R = Me) was stable under these conditions, a phenol coupling reaction is presumably involved. Since a surface culture of *A. flavus* undergoes the same colour change with time, the nature of the yellow pigment contained in the spore-heads was investigated. This was shown to be, not asperflavin, but, the relatively stable substituted



(X)



(XI)



(XII)

gentisyl aldehyde, aspergin (XII; R = n-C_5H_{11}·CH:CH·), recently isolated from an unidentified *Aspergillus* sp.[9] Although the analytical data and physical constants of the crude pigment agreed closely with those recorded for aspergin, the mass spectrum showed a second molecular ion at *m/e* 304 consistent with the presence of the dihydro-derivative, flavoglaucin (XII; R = n-C_7H_{15}). This was confirmed by the n.m.r. spectrum, in which signals from protons in close proximity to the disub-

[4] A. Mahmoodian and C. E. Stickings, *Biochem. J.*, 1964, **92**, 369.

[5] Y. Yamamoto, N. Kiriyama, and S. Arakata, *Chem. and Pharm. Bull.* (*Japan*), 1968, **16**, 304.

[6] J. Atherton, B. W. Bycroft, J. C. Roberts, P. Roffey, and M. E. Wilcox, *J. Chem. Soc.* (*C*), 1968, 2560.

[7] M. N. Koslov, S. A. Popravko, and M. M. Shemyakin, *Doklady Akad. Nauk S.S.S.R.*, 1963, **150**, 1285.

[8] A. G. Perkin and J. J. Hummel, *J. Chem. Soc.*, 1894, **65**, 923.

[9] L. B. Sokolov, L. E. Alekseeva, V. A. Kulbakh, N. A. Kuzenetsova, and V. S. Nyn, *Antibiotiki*, 1971, **16**, 504.

stituted ethylenic double bond appeared as doublets, instead of the expected singlets, indicative of the presence of two closely-related molecular species. The identity with flavoglaucin was confirmed by comparison with the spectrum of an authentic specimen.

Flavoglaucin has been recorded as a metabolic product of a large number of *Aspergillus* sp.,[10,11] particularly of the *A. glaucus* series, to which at one time [10] it was considered to be specific; it has not previously been isolated from *A. flavus*. Several groups of workers [10,12,13] have commented on the difficulty of separating flavoglaucin from more unsaturated contaminants. The antimicrobial activities of these pigments will be recorded elsewhere.

The u.v. ($\lambda_{max.}$ 260 and 297 nm) and i.r. spectra ($\nu_{max.}$ 1725 cm^{-1}) of the phenolic metabolite shown by high-resolution mass spectroscopy to have the composition $C_9H_8O_4$ suggested that the compound was a 7-hydroxyphthalide.[14] This assignment was confirmed by the formation of an intense green colour with iron(III) chloride, by the n.m.r. spectrum of the compound which contained a two-proton singlet at τ 4·8, and by the mass spectrum which showed peaks due to a prominent fragment ions at *m/e* 151 (M^+ — CHO) and 122 (M^+ — $C_2H_2O_2$).[15]

The 3-methylene group of the phthalide ring and the 7-hydroxy-group account for three of the eight protons in the molecule. The remaining five protons were contained in a methyl group (three-proton singlet at τ 7·95), the aromatic ring (one-proton singlet at τ 3·6), and a hydroxy-group ($\nu_{max.}$ 3425 cm^{-1}), which also accounts for the fourth oxygen atom. It follows that substituents are present at two of the three positions remaining on the 7-hydroxyphthalide nucleus. On biogenetic considerations the hydroxy-group should be placed at position 5, but the methyl group could be at position 4 or 6 giving structures (VIII; $R^1 = R^2 = H$) or (IX; $R^1 = R^2 = H$).

Comparison (Table 2) of the spectroscopic data reported in the literature [16-19] for the model phthalides (VIII) and (IX) suggested that in the n.m.r. spectra the methyl group and aromatic ring proton were more shielded in the 4-methyl series; and that the 4-methyl compound showed a small (5—10 nm) bathochromic shift of the long-wavelength u.v. maximum compound with the 6-methyl series.

Data obtained for the $C_9H_8O_4$ compound agreed closely with the 4-methyl series. Methylation with methyl iodide in acetone in the presence of potassium carbonate gave a dimethyl ether, m.p. 203°, identical with the dimethyl ether (VIII; $R^1 = R^2 = Me$) obtained by methylation under the same conditions of the synthetic 7-hydroxyphthalide (VIII; $R^1 = Me$, $R^2 = H$).[16]

TABLE 2

U.v. absorption data (for methanolic solutions) and chemical shifts (τ values) for protons in 4-methyl- and 6-methyl-5,7-dihydroxyphthalides and their methyl ethers

Compound	Solvent	ArH	3-H	Me	OMe	$\lambda_{max.}$/nm	ε	Ref.
4-Me								
(VIII; $R^1 = R^2 = H$)	CD_3·OD	3·62	4·8	7·95		260, 297	9500, 4000	
(VIII; $R^1 = Me$, $R^2 = H$)	$CDCl_3$	3·55	4·81	7·96	6·12	258, 298	13,200, 6100	16
(VIII; $R^1 = R^2 = Me$)	$CDCl_3$	3·52	4·84	7·92	5·96, 6·00	260, 297	14,700, 7500	
6-Me								
(IX; $R^1 = H$, $R^2 = Me$)						260, 290	13,000, 2800	17, 18
(IX; $R^1 = Me$, $R^2 = H$)	$CDCl_3$	3·27	4·79	7·90	6·12	259, 293	14,000, 2750	18, 19
(IX; $R^1 = R^2 = Me$)	$CDCl_3$	3·36	4·82	7·85	6·0, 6·1	259, 288	14,000, 2200	17, 19

EXPERIMENTAL

M.p.s were taken on a Kofler hot-stage apparatus and are corrected. Unless otherwise stated, i.r. spectra were determined for mulls in Nujol, and u.v. spectra and optical rotations for solutions in methanol. N.m.r. spectra were obtained at 100 MHz with tetramethylsilane as internal standard. Molecular weights were taken from the parent peaks in the low resolution mass spectra. Light petroleum had b.p. 60—80°. Merck silica gel G_{254} was used in thin layer chromatography (t.l.c.).

Isolation of Asperflavin.—The *Aspergillus flavus* strain was grown in surface culture on Raulin–Thom medium. The fermentation was harvested (7 l) and the chromatographic separation of the asperentins was carried out as previously described.[1] Fractional crystallisation from ethyl acetate of the residues (80 mg) after separation of 5′-hydroxyasperentin gave citrine prisms (9 mg; 1·3 mg l^{-1}), $[\alpha]_D^{20}$ +4° (*c* 0·3), m.p. 225—230°, resetting to needles which decomposed at *ca.* 280° without melting, of *asperflavin* [(+)-3,4-*dihydro*-3,6,9-*trihydroxy*-8-*methoxy*-3-*methylanthracen*-1(2H)-*one*] (I; R = H) (Found: C, 66·4; H, 5·75%; *M*, 288. $C_{16}H_{16}O_5$ requires C, 66·7; H, 5·6%; *M*, 288], $\lambda_{max.}$ 230, 269, 317, 335, and 392 nm (log ε 4·13, 4·51, 3·64, 3·40, and 3·91), $\nu_{max.}$ 3490, 3350, 1632, and 1582 cm^{-1}. It was insoluble in sodium hydrogen carbonate, but dilute solutions in sodium carbonate had an intense yellowish green fluorescence. In 2N-sodium hydroxide it gave a yellow solution which rapidly

[10] B. S. Gould and H. Raistrick, *Biochem. J.*, 1934, **28**, 1640.
[11] A. Quilico and L. Panizzi, *Ber.*, 1943, **76**, 348.
[12] H. Raistrick, R. Robinson, and A. R. Todd, *J. Chem. Soc.*, 1937, 80.
[13] A. J. Birch, A. J. Ryan, J. Schofield, and H. Smith, *J. Chem. Soc.*, 1965, 1231.
[14] L. A. Duncanson, J. F. Grove, and J. Zealley, *J. Chem. Soc.*, 1953, 1331.
[15] E. Stenhagen, S. Abrahamsson, and F. W. McLafferty, 'Atlas of Mass Spectral Data,' Vol. 1, Interscience, 1969, p. 649.
[16] A. J. Birch and J. J. Wright, *Austral. J. Chem.*, 1969, **22**, 2635.
[17] A. N. Starratt, *Canad. J. Chem.*, 1968, **46**, 767.
[18] P. J. Aucamp and C. W. Holzapfel, *J. S. African Chem. Inst.*, 1968, **21**, 26.
[19] A. J. Birch and R. A. Russell, *Austral. J. Chem.*, 1971, **24**, 1975.

became orange. Dilute solutions in organic solvents showed a green fluorescence. It gave an intense dark green colour with iron(III) chloride in ethanol. Sublimation at 160° and 10^{-1} mmHg gave a solid which crystallised from ethyl acetate in needles (decomp. 290° without melting) of anhydroasperflavin (see later), identified by the i.r. spectrum. Asperflavin was unstable in light and air. Solutions in organic solvents slowly became dark green, whilst thin layers of silica impregnated with asperflavin rapidly changed colour from citrine to green. The green product remained at the origin when the t.l.c. plate was developed in chloroform–methanol (97 : 3) (asperflavin R_F 0·13).

The 6-O-*methyl ether* (I; R = Me), prepared with an excess of methyl iodide heated under reflux in acetone during 8 h in the presence of anhydrous potassium carbonate, crystallised from methanol or ethyl acetate in citrine needles, m.p. 225° (decomp.) (Found: C, 67·0; H, 6·1%; *M*, 302. $C_{17}H_{18}O_5$ requires C, 67·5; H, 6·0%; *M*, 302), $\lambda_{max.}$ 228, 269, 315, 327, and 386 nm (log ε 4·22, 4·65, 3·77, 3·67, and 4·03), $\nu_{max.}$ 3440, 1635, 1600, and *ca.* 1580 cm^{-1}. It was stable in air and light and gave an intense green colour with iron(III) chloride in ethanol.

Isolation of Anhydroasperflavin and 5,7-Dihydroxy-4-methylphthalide. The culture filtrate (7 l), after extraction by chloroform,[1] was re-extracted with ethyl acetate (2 × 1 l). Recovery afforded a dark brown resin (0·66 g), which did not yield any solid on trituration with acetone,[1] but which showed specific absorption at 268 nm. It was chromatographed in benzene–methanol (98 : 2) on silica (Merck 7734, 30 g, 18 × 1·8 cm). Elution of an orange band with the same solvent (150 ml) gave a brown resin (22 mg), which crystallised from ethyl acetate in yellow needles (2 mg, 0·3 mg l^{-1}), decomposing without melting at 290°, of *anhydroasperflavin* (1,6-*dihydroxy*-8-*methoxy*-3-*methyl*-9-*anthrone*) (III; $R^1 = R^3 = H$, $R^2 = Me$) (Found: *M*, 270·0887. $C_{16}H_{14}O_4$ requires *M*, 270·0892), $\lambda_{max.}$ 221, 250, 270, 295, and 340 nm (log ε 4·02, 3·95, 3·91, 3·87, and 4·17), $\nu_{max.}$ 3260, 1640, 1615, 1587, 1350, 1262, 1240, 1220, 1200, 1172, 1160, 1138, 1092, 975, 958, 930, 905, 860, 840, and 808 cm^{-1}. Prisms ($\nu_{max.}$ 3320, 1640, 1615, 1590, 1345, *ca.* 1270, 1248, 1220, 1178, 1160, 1140, 1092, 975, 958, 930, 905, 860, 838, and 817 cm^{-1}) were sometimes obtained from the same solvent.

Anhydroasperflavin gave a green colour with iron(III) chloride and an orange solution, which became red on standing, with 2N-sodium hydroxide. It was unstable in air and light giving greenish brown products. Unlike the isomeric anthrone (III; $R^1 = Me$, $R^2 = R^3 = H$), it was only sparingly soluble in organic solvents and almost insoluble in chloroform.

The *triacetate* (1,6,9-*triacetoxy*-8-*methoxy*-3-*methylanthracene*) (II; $R^1 = R^3 = Ac$, $R^2 = Me$), prepared with acetic anhydride in pyridine during 18 h at room temperature, crystallised from methanol in yellow prisms, m.p. 225° (decomp.) (Found: *M*, 396. $C_{22}H_{20}O_7$ requires *M*, 396), $\nu_{max.}$ 1762, 1630, and 1570 cm^{-1}, $\lambda_{max.}$ 224, *ca.* 243, 258, 376, 379, and 400 nm (log ε 4·26, 4·61, 5·01, 3·78, 3·78, and 3·68).

It was unstable and was rapidly oxidised in air to orange-red products.

Continued elution of the column with benzene–methanol (95 : 5, 125 ml) brought off a pale yellow band giving a brown resin (24 mg). It crystallised from ethyl acetate in prisms (2 mg, 0·3 mg l^{-1}), which sublimed with decomposition at 220—240° [m.p. (sealed tube, uncorr.), 242°]. Further purification by sublimation at 150° and 10^{-2} mmHg followed by crystallisation from ethyl acetate gave 5,7-*dihydroxy*-4-*methylphthalide* (VIII; $R^1 = R^2 = H$) (Found: *M*, 180·0434. $C_9H_8O_4$ requires *M*, 180·0423), $\lambda_{max.}$ *ca.* 227, 260, and 297 nm (ε 15,800, 9400, and 4100), $\nu_{max.}$ 3425, 3340, 1725, 1630, and 1532 cm^{-1}. It was insoluble in sodium hydrogen carbonate, but dissolved in sodium carbonate and in 2N-sodium hydroxide. It gave an intense green colour with iron(III) chloride in ethanol.

The phthalide (0·3 mg) was heated under reflux for 5 h in acetone with an excess of methyl iodide in the presence of anhydrous potassium carbonate (1 mg). Evaporation *in vacuo* and trituration of the residue with water afforded prisms of the dimethyl ether (VIII; $R^1 = R^2 = Me$), m.p. 200—203° (from methanol) (see later), identified by comparison of the i.r. spectra.

1,6-*Dihydroxy*-8-*methoxy*-3-*methyl*-9-*anthrone* (*Anhydroasperflavin*) (III; $R^1 = R^3 = H$, $R^2 = Me$).—(*a*) *By reduction of questin.* 1,6-Dihydroxy-8-methoxy-3-methylanthraquinone (questin) (VI; $R^1 = H$, $R^2 = Me$)[4] (4 mg) in glacial acetic acid (1 ml) was heated under reflux for 15 min with zinc powder (20 mg). The cooled solution was filtered and the filtrate was diluted with water (2 ml). The resulting precipitate was collected and recrystallised from ethyl acetate giving prisms (3 mg), which charred without melting at 280°, of 1,6-*dihydroxy*-8-*methoxy*-3-*methyl*-9-*anthrone* (III; $R^1 = R^3 = H$, $R^2 = Me$) (Found: C, 71·4; H, 5·0. $C_{16}H_{14}O_4$ requires C, 71·1; H, 5·2%). The i.r. spectrum was identical with that of anhydroasperflavin (prism form).

Acetylation with acetic anhydride in pyridine during 18 h at room temperature gave prisms, m.p. and mixed m.p. 225° (decomp.), of the triacetate (II; $R^1 = R^3 = Ac$, $R^2 = Me$), identical (i.r.) with triacetylanhydroasperflavin.

(*b*) *From asperflavin.* Asperflavin (6 mg) in acetic acid (0·3 ml) containing concentrated hydrochloric acid (0·03 ml) was heated at 100° for 30 min. The precipitate (2 mg) was filtered off and identified as anhydroasperflavin by comparison of the i.r. spectra. Anhydroasperflavin (3 mg) was recovered from the filtrate by dilution with water and crystallisation of the crude precipitate from ethyl acetate.

Acetylation of Asperflavin.—Asperflavin (5 mg) in pyridine (0·1 ml) and acetic anhydride (0·2 ml) was set aside at room temperature for 18 h. After volatile components had been removed *in vacuo*, the residual gum deposited prisms (0·5 mg), m.p. 215° (decomp.) (from methanol), of the triacetate (II; $R^1 = R^3 = Ac$, $R^2 = Me$), identified by the i.r. spectrum. The residue was recovered and purified by p.l.c. [silica (10 × 20 × 0·1 cm), chloroform–methanol (97 : 3)]. The material from a yellow band (R_F 0·3) was recovered and crystallised from methanol in citrine needles (1 mg), m.p. 198° (decomp.), of the *acetate* (I; R = Ac) (Found: C, 65·2; H, 5·7%; *M*, 330. $C_{18}H_{18}O_6$ requires C, 65·4; H, 5·5%; *M*, 330), $\nu_{max.}$ 3480, 1780, 1635, 1608, and 1582 cm^{-1}, $\lambda_{max.}$ 218, *ca.* 259, 266, 294, 305, 319, 388, and 404 nm (log ε 4·38, 4·66, 4·77, 3·76, 3·78, 3·57, 4·03, and 4·00). Bands visible under u.v. light (254 nm) at R_F 0·4, 0·6, and 0·8 yielded only trace amounts of intractable material.

1,8-*Dihydroxy*-6-*methoxy*-3-*methyl*-9-*anthrone* (III; R^1 = Me, $R^2 = R^3 = H$) (*'physcion anthranol B'*).—This was obtained by reduction of physcion,[3] and had m.p. 181° (from benzene) (lit.,[3] 181—182°), $\nu_{max.}$ 1625 and 1600 cm^{-1}, $\lambda_{max.}$ 225, 272, 303, and 355 nm (log ε 3·98, 4·04, 3·98, and 4·24). It was readily soluble in chloroform, $\lambda_{max.}$ identical above 250 nm with the spectrum in methanol.

Acetylation of the Anthrone (III; $R^1 = Me$, $R^2 = R^2 =$ H).—(*a*) The anthrone (5 mg) was acetylated as described

before. The residual gum was triturated with water and furnished yellow prisms (4 mg), m.p. 275—276° (from methanol), of 1,8,9-*triacetoxy*-10-*acetyl*-6-*methoxy*-3-*methylanthracene* (V) (Found: C, 66·0; H, 5·0%; *M*, 438. $C_{24}H_{22}O_8$ requires C, 65·7; H, 5·1%; *M*, 438), $\nu_{max.}$ 1770, 1700, 1630, and 1570 cm^{-1}, $\lambda_{max.}$ *ca.* 227, *ca.* 245, *ca.* 255, 264, 348, 367, 390, and 410 nm (log ε 4·15, 4·39, 4·81, 5·07, 3·53, 3·67, 3·72, and 3·64).

(*b*) The anthrone (5 mg) was boiled with acetic anhydride for 6 h.[8] Recovery afforded the *triacetate* (II; R^1 = Me, R^2 = R^3 = Ac), m.p. 235°, $\nu_{max.}$ 1770, 1625, and 1570 cm^{-1}, $\lambda_{max.}$ 225, *ca.* 243, 263, 368, and 398 nm (log ε 4·22, 4·39, 4·99, 3·61, and 3·58).

5,7-*Dimethoxy*-4-*methylphthalide*.— 7-Hydroxy-5-methoxy-4-methylphthalide (15 mg), m.p. 215—216° (lit.,[16] 216—218°), in acetone (2 ml) with anhydrous potassium carbonate (30 mg) was heated under reflux with an excess of methyl iodide for 5 h. The mixture was evaporated to dryness and the residue was triturated with water. The insoluble neutral product was filtered off and starting material (6 mg), identified by the i.r. spectrum, was recovered by acidification of the filtrate with dilute hydrochloric acid. The neutral product was dried and twice recrystallised from methanol giving prisms (6 mg), m.p. 203°, of 5,7-*dimethoxy*-4-*methylphthalide* (VIII; R^1 = R^2 = Me) (Found: C, 63·2; H, 5·7. $C_{11}H_{12}O_4$ requires C, 63·4; H, 5·8%), $\nu_{max.}$ 1740, 1625, and 1610 cm^{-1}, $\lambda_{max.}$ 260 and 297 nm (ε 14,700 and 7500).

5,7-Dimethoxy-6-methylphthalide (IX; R^1 = R^2 = Me), m.p. 170° (lit.,[17,18] 172—173°) had $\nu_{max.}$ 1745 and 1610 cm^{-1}.

Isolation of Pigments from the Spore-heads of A. flavus.— A Czapek–agar slope contained in a Roux bottle was seeded with the *A. flavus* culture in the usual way and incubated at 25°. After 6 days the yellow spore-bearing surface was washed with chloroform (20 ml). Recovery gave a yellow resin (20 mg), which was purified by p.l.c. [silica (20 × 20 × 0·1 cm), chloroform–methanol (97 : 3)]. The material contained in the principal yellow band (R_F 0·61) was recovered and recrystallised three times from light petroleum giving needles (7 mg), m.p. 95—96°, of aspergin (XII; R = n-C_5H_{11}·CH:CH·) (Found: C, 76·0; H, 8·6%; *M*, 302. Calc. for $C_{19}H_{26}O_3$: C, 75·5; H, 8·7%; *M*, 302), $\nu_{max.}$ 3260, 1625, and 1590 cm^{-1}, $\lambda_{max.}$ 230, 270, *ca.* 310, and 398 nm (log ε 4·18, 4·11, 3·75, and 3·78). The i.r. spectrum was identical with that recorded for aspergin, m.p. 91—92°;[9] the n.m.r. spectrum showed the signals recorded[9] for aspergin at τ −1·90 (chelated OH), −0·10 (CHO), 2·95 (ArH), 3·4—4·0 (1″- and 2″-H_2), 4·65 (2′-H), 5·00 (OH), 6·65 (1′-H_2), 7·7 (3″-H_2), 8·20 and 8·26 (=CMe_2), 8·66 (CH_2), and 9·10 (Me); together with signals at τ −1·75 (chelated OH), −0·25 (CHO), 3·15 (ArH), 5·50 (OH), and 7·05 [1-H_2 (*cf.* ref. 9)] attributed to flavoglaucin (XII; R = n-C_7H_{15}).

Aspergin did not become green on exposure to air and light. With iron(III) chloride in ethanol it gave a transient greenish brown colour.

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